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Fluorescent Exendin-4 Derivatives for Pancreatic β-Cell Analysis

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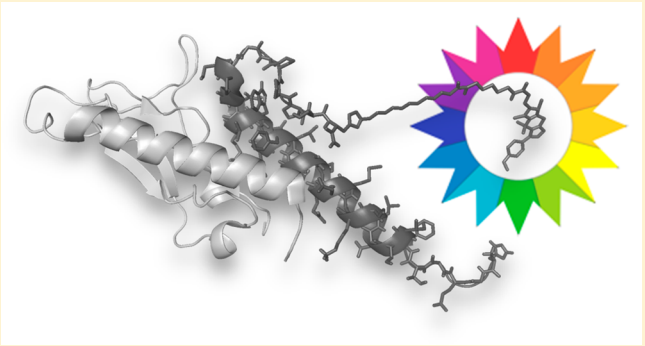
ABSTRACT: The ability to reliably identify pancreatic β-cells would have far reaching implications for a greater understanding of β-cell biology, measurement of β-cell mass in diabetes, islet transplantation, and drug development. The glucagon-like peptide-1 receptor (GLP1R) is highly expressed on the surface of insulin producing pancreatic β-cells. Using systematic modifications of the GLP1R ligand, exendin-4, we screened over 25 compounds and identified a palette of fluorescent exendin-4 with high GLP1R binding affinity. We show considerable differences in affinity, as well as utility of the top candidates for flow cytometry and microscopy of β-cells. Some of the developed compounds should be particularly useful for basic and translational β-cell research.



INTRODUCTION

The cell surface expressed glucagon-like peptide-1 receptor (GLP1R) has emerged as a high value drug target in diabetes.[1] GLP1R is highly expressed on insulin producing pancreatic β-cells, but unfortunately there is a lack of suitable antibodies that could be used for reliable identification.[2] The fortuitous discovery of GLP1R peptide antagonists from the gila monster has led to the development of different exendin-4 (E4) derived therapeutics, many with nanomolar affinity.[1,3] In addition, certain E4 derivatives are being developed for whole body nuclear imaging[4−7] or intraoperative imaging.[8] Given the high affinity of E4 ligands we reasoned that they could also be optimized and developed for flow cytometry and microscopy applications; however, the literature on this topic is sparse. Furthermore, there is a lack of structure−activity data when it comes to E4 neopeptides, linkers, and fluorochrome modifications. We therefore conducted the current study to broadly address the following questions: (i) what is the ideal E4-like neopeptide sequence that allows for quick, efficient fluorescent conjugation while preserving GLP1R affinity; (ii) which position on the ideal E4-like neopeptide best tolerates fluorophore conjugation; (iii) can a palette of fluorochromes be developed so that E4 derivatives can be used for multicolor flow analysis of β-cells?

Since the effects of chemical modifications on the imaging behavior of E4 are poorly understood, we systematically designed an ideal E4-like neopeptide by exploring the effect of amino acid substitution/addition (K, R, Pra (propargylglycine)) at different modification sites (positions 12, 27, 40) and then examined the effect of a variety of fluorochromes (Pacific blue (PB), BODIPY FL (BF), BODIPY TMR-X (BTMR), Texas Red-X (TR), CyAl 5 (Cy), VT680 XL (V6), and VT750 S (V7)) on GLP1R binding affinity (Figure 1). We synthesized 19 new derivatives, tested them in competitive receptor binding assays, and then confirmed properties by flow cytometry and microscopic imaging. Based on these results, we made some unexpected observations and present a palette of fluorescent E4 derivatives uniquely for microscopy and flow cytometry.

EXPERIMENTAL PROCEDURES

Materials. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted and used without further purification. Exendin-4 (1−39) amide (E4) was obtained from Amlyin/Eli Lilly (San Diego, CA, USA). FMOC-R^{12}E4 (molecular weight 4437, FMOC-HGEGTFTSDLSRQMEEEAVRLFIEWLKN GGPSSGAPPPS), FMOC-R^{27}E4 (molecular weight 4437, FMOC-HGEGTFTSDLSKQMEEEAVRLFIEWLRNGGPSSGAPPPS), FMOC-$K^{40}R^{12,27}$E4 (molecular weight 4594, FMOC-HGEGTFTSDLSRQMEEEAVRLFIEWLRNGGPSSGAPPPSK), Pra^{12}E4 (molecular weight 4155, HGEGTFTSDLSPraQMEEEAVRLFIEWLKNGGPSSGAPPPS), Pra^{27}E4 (molecular weight 4154, HGEGTFTSDLSKQMEEEAVRLFIEWLPraNGGPSSGAPPPS), and Pra^{40}E4 (molecular weight 4282, HGEGTFTS-

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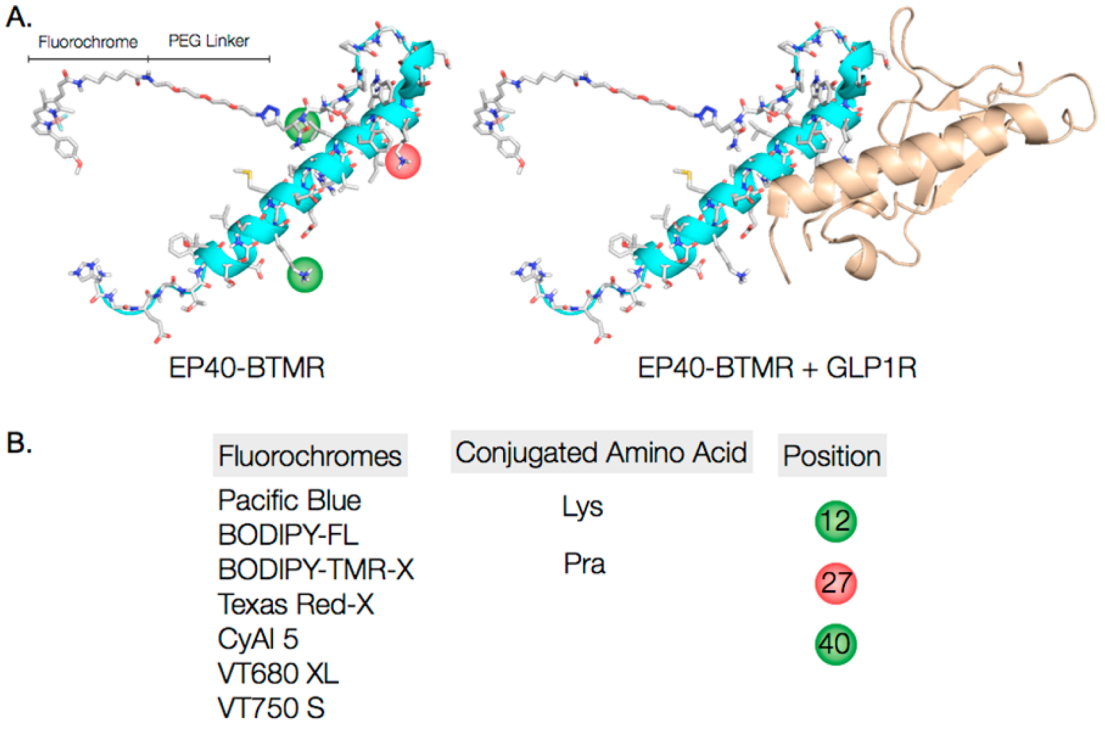

Figure 1. A. Left: Molecular model of an exendin-4 like neopeptide conjugated to BODIPY TMR-X at the 40 position, EP40-BTMR, based on the NMR structure, 1JRJ. Right: Molecular model of EP40-BTMR complexed with the extracellular domain of GLP1R is based on the crystal structures 3C5T and 1JRJ. B. A palette of fluorescent exendin-like neopeptides were developed by examining fluorochromes, different amino acid chemistry and position.

DLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSPra) all with c-terminal amidation were purchased from CSBio (Menlo Park, California). Fluorochromes Pacific Blue (PB), BODIPY FL(BF), BODIPY TMR-X (BTMR), and Texas Red-X (TR) were purchased as the *N*-hydroxysuccinimide (NHS) esters from Invitrogen. VT680 XL (V6) and VT750 S (V7) were purchased as NHS esters from Perkin-Elmer. CyAl 5 (Cy) NHS ester was prepared as previously described.[9] LC-ESI-MS analysis was performed on a Waters (Milford, MA) LC-MS system. Reverse phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1200 Series with a Poroshell 120 EC-C18 (4.6 × 50 mm, 2.7 μm) reversed-phase column. HPLC mobile phases used for purification were A: 0.1% trifluoroacetic acid (TFA) in deionized water and B: acetronitrile. MALDI-MS spectra were collected on a Voyager-DE Biospectrometry workstation spectrometer (Applied Biosystems, Foster City, CA). The matrix used for MALDI-MS was 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in dH_2O/CH_3CN (1:1 v/v) containing 0.1% TFA. Samples were mixed with matrix 1:1.

Preparation of EK12-BF (8), EK27-BF (9), and EK40-BF (10). BODIPY FL-NHS (17.5 μL, 2 equiv, 10 mg/mL in dimethylsulfoxide (DMSO) containing 0.3% triethylamine (TEA)) was reacted with 100 μL of FMOC-E4 (EK12, EK27, or EK40 at 10 mg/mL in DMSO) at room temperature (RT) with gentle mixing for 30 min. The lysine conjugated E4 was RP-HPLC purified using a linear gradient (25−100% B from 0.3 to 10 min) at a constant flow of 1.5 mL/min. Peaks were monitored at 254 and 504 nm UV detector. Eluting peaks were collected separately, concentrated by rotary evaporation, and redissolved in DMSO with 3% TEA to remove FMOC from the N-terminus. After 12 h, the deprotected product was isolated by the above-described HPLC method. The final product was concentrated by rotary evaporation to remove solvents and redissolved in 1×PBS. Digestion of the conjugates was accomplished by the addition of a trypsin solution (10 μL, 1 mg/mL) and incubation at 37 °C for 3 h. Analysis of the trypsin digested samples was accomplished by MALDI-MS analysis. A bicinchoninic acid assay (BCA) was conducted to determine yield.

Preparation of EK12-V7(11) and EK40-V7(12). To a 100 μL (10 equiv) stirring solution of VT750 S-NHS (10 mg/mL in DMSO), 50 μL of FMOC-E4 (EK12 or EK40 at 7.8 mg/mL in DMSO with 1 μL TEA) was added and allowed to react for 2 h. The crude reaction mixture was purified with a two-step RP-HPLC monitored at 280 and 750 nm, and a step gradient of 5−35% B 0−3 min and 35−45% 3−13 min was necessary for HPLC isolation. The final product was concentrated by rotary evaporation to remove the organic solvents and redissolved in 1×PBS. A BCA assay was conducted to determine yield.

Preparation of Azide-Functionalized Fluorophore. To a 100 μL (1 equiv) solution of fluorophore (10 mg/mL in DMSO), 11-azido-3,6,9-trioxaun-decan-1-amine (10 equiv) was added. The reaction was stirred for 5 min at RT. Azide functionalized fluorophores were isolated with a linear gradient (PB, BTMR, Cy, V7: 5−100% B 0.3−10 min; BF: 25−100% B 0.3−12 min) at a flow rate of 2.5 mL/min. Azide-TR and Azide-V6 were purified with a C18 semipreparative column at a flow rate of 5.0 mL/min and linear gradient (TR:15−95% B 0.3−10 min; VT6: 5−15% B 0−0.3 min, 15−95% B over 0.3−10 min). The isolated products were concentrated by rotary evaporation and stored at −20 °C until use.

Preparation of EP12-FL (13,15,17,19,21,23,25) and EP40-FL (14,16,18,20,22,24,26). All fluorescent-derivatized EP12 and EP40 conjugates were synthesized using a previously described method with slight modifications.[10] Briefly, to a

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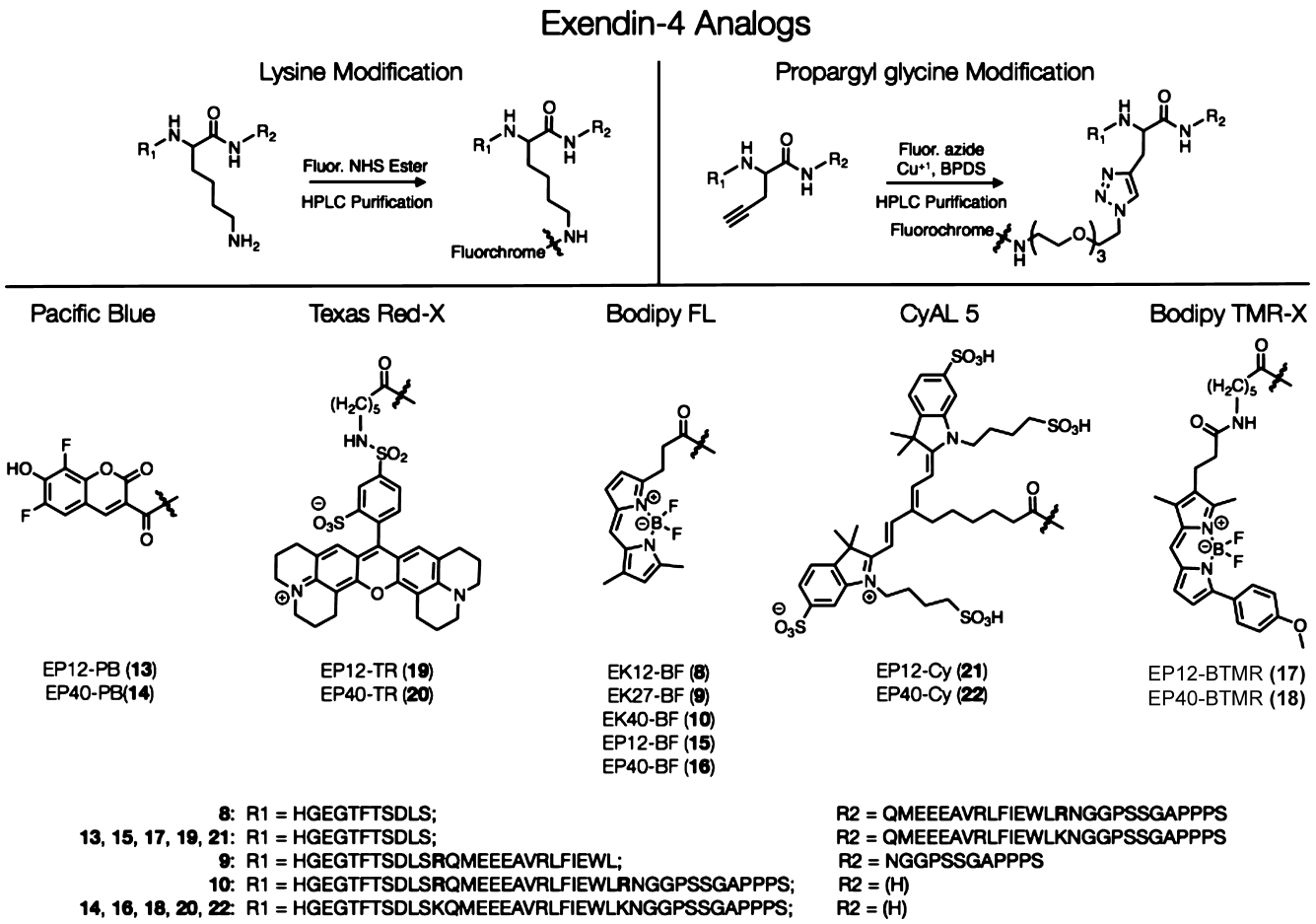

Figure 2. Experimental strategy for fluorescent exendin-like neopeptide conjugation (the structure of the commercially available fluorochromes VT680 XL and VT750 S are proprietary and have not been published).

microwave vessel 90 μL (60 equiv) of bathophenanthroline disulfonate (80 mM in water) and 113.1 μL (46 equiv) of tetrakis(acetonitrile)copper(I) hexafluorophosphate (50 mM in MeCN) were added, followed by a mixture of EP12 or EP40 (1 mM in 1×PBS) and azide-functionalized fluorophore (25 mM in 25% 100 mM sodium bicarbonate/75% MeCN for PB; MeCN for BF; 25% MeCN/75% DMF for BTMR; 20% DMF/80% MeCN for TR; deionized water for Cy, V6, V7). The vessel was purged with argon and capped prior to microwave irradiation (60 °C, 30 W) for 5 min. The mixture was filtered through a 0.22 μm spin filter and isolated by RP-HPLC with a linear gradient (PB, Cy, V6, V7: 5−100% B 0.3−10 min; BF: 25−100% B 0.3−12 min; BTMR: 25−100% B 0.3−10 min; TR: 5−95% B 0.3−10 min) at a flow rate of 2.5 mL/min. The final product was concentrated by rotary evaporation to remove the organic solvents and redissolved in 1×PBS. A BCA assay was conducted to determine yield for PB, BF, Cy, V6, and V7 conjugates. A Nanodrop 1000 was used to determine yield of BTMR and TR conjugates, due to overlapping absorbances with the BCA assay.

Cell Culture. Human embryonic kidney (HEK-293) cells stably expressing the human GLP-1 receptor (HEK-hGLP1R) were grown in high-glucose DMEM containing 10% (v/v) heat-inactivated FBS, 50 U/mL penicillin, 10 μg/mL streptomycin, 1 mM sodium pyruvate, and 150 μg/mL G418 (Geneticin). GLP1R−negative HEK-293 (ATCC) were grown in high-glucose DMEM containing 10% (v/v) heat-inactivated FBS, 50 U/mL penicillin, and 10 μg/mL streptomycin. Mouse insulinoma NIT-1 cells were grown in F-12K containing 10% (v/v) heat-inactivated FBS, 1% sodium bicarbonate, 50 U/mL penicillin, and 10 μg/mL streptomycin. β-Cells were also analyzed from C57BL/6 mice using flow cytometry gating on the autofluorescence using an established procedure.[11] Cells were imaged by microscopy after seeding in a 96 well plate at 5.5×10^4 cells per well and incubation with or without fluorescent exendin-4 (200 nM). After 1 h, cells were washed twice with HBSS solution and imaged (Applied Precision DeltaVision with an Olympus IX70 inverted microscope).

In Vitro Receptor Binding Assay. The receptor binding affinities of E4 analogues were determined in HEK-hGLP1R using a slight modification of a previously described method.[12] Cells were seeded in a 96 well plate at 5.5×10^4 cells per well, grown for 48 h, washed twice with binding buffer (120 mM NaCl, 1.2 mM $MgSO_4$, 13 mM sodium acetate, 5 mM KCl, 1.2 g/L Tris, 2 g/L bovine serum albumin (BSA), and 1.8 g/L glucose, pH 7.6), and cotreated with 30 pM of ^{125}I-exendin-4 (9−39, PerkinElmer, Boston, MA) and unlabeled exendin-4 or fluorescent exendin-4 analogues (final concentration range: 10^{-12}−10^{-6} M). After incubating for 2 h at 37 °C, the cells were washed three times with chilled PBS containing 1 mg/mL BSA, lysed (RIPA 1× buffer, 15 min) and ^{125}I contents were measured using a Wallac Wizard 3″ 1480 Automatic Gamma Counter.

Flow Cytometry. HEK-hGLP1R, HEK-293, and NIT-1 cells were seeded in a 6 well plate at 1.0×10^6 cells per well, grown for 24 h, washed with HBSS, and then incubated at 37

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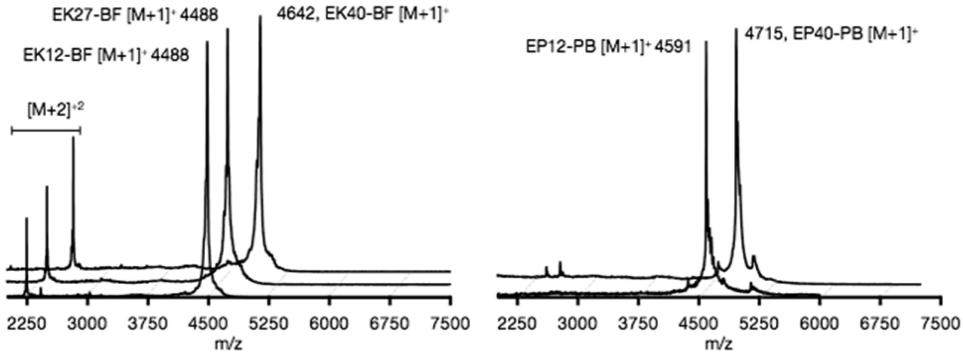

Figure 3. Representative MALDI-MS spectra of fluorescent exendin-4-like neopeptides.

Table 1

compound	parent peptide	conjugation site	fluorophore	short name	binding affinity (IC_{50}, nM)
1	Exendin-4(1−39) amide	None	None	E4	3.2 ± 1.3
2	R^{27} E4	None	None	EK12	4.8 ± 1.5
3	R^{12} E4	None	None	EK27	2.2 ± 1.3
4	$R^{12,27}$ K^{40} E4	None	None	EK40	6.1 ± 1.4
5	Pra^{12} E4	None	None	EP12	4.2 ± 1.4
6	Pra^{27} E4	None	None	EP27	12.5 ± 1.4
7	Pra^{40} E4	None	None	EP40	3.3 ± 1.3
8	R^{27} E4	K^{12}	BODIPY FL	EK12-BF	16.9 ± 1.3
9	R^{12} E4	K^{27}	BODIPY FL	EK27-BF	35.7 ± 1.3
10	$R^{12,27}$ K^{40} E4	K^{40}	BODIPY FL	EK40-BF	19.9 ± 1.2
11	R^{27} E4	K^{12}	VT750 S	EK12-V7	61.6 ± 1.2
12	$R^{12,27}$ K^{40} E4	K^{40}	VT750 S	EK40-V7	96.1 ± 1.3
13	Pra^{12} E4	Pra^{12}	Pacific Blue	EP12-PB	3.0 ± 1.4
14	Pra^{40} E4	Pra^{40}	Pacific Blue	EP40-PB	4.1 ± 1.2
15	Pra^{12} E4	Pra^{12}	BODIPY FL	EP12-BF	4.3 ± 1.3
16	Pra^{40} E4	Pra^{40}	BODIPY FL	EP40-BF	3.9 ± 1.1
17	Pra^{12} E4	Pra^{12}	BODIPY TMR-X	EP12-BTMR	2.3 ± 1.3
18	Pra^{40} E4	Pra^{40}	BODIPY TMR-X	EP40-BTMR	3.6 ± 1.3
19	Pra^{12} E4	Pra^{12}	Texas Red-X	EP12-TR	5.1 ± 1.3
20	Pra^{40} E4	Pra^{40}	Texas Red-X	EP40-TR	6.4 ± 1.3
21	Pra^{12} E4	Pra^{12}	CyAl 5	EP12-Cy	6.4 ± 1.2
22	Pra^{40} E4	Pra^{40}	CyAl 5	EP40-Cy	3.9 ± 1.3
23	Pra^{12} E4	Pra^{12}	VT680 XL	EP12-V6	115 ± 1.2
24	Pra^{40} E4	Pra^{40}	VT680 XL	EP40-V6	91.8 ± 1.2
25	Pra^{12} E4	Pra^{12}	VT750 S	EP12-V7	2.6 ± 1.1
26	Pra^{40} E4	Pra^{40}	VT750 S	EP40-V7	0.9 ± 1.2

°C in binding buffer with or without fluorescent exendin-4 (10−200 nM). After 1 h, cells were washed twice with HBSS solution and harvested by trypsin-EDTA. The cells were washed with binding buffer, centrifuged, and resuspended in PBS with 2% BSA. The cell suspension was immediately analyzed using a LSRII flow cytometer (BD Bioscience). Forward angle light scattering and side angle light scattering were recorded as well as pacific blue, FITC for BF, PE for BTMR, Alexa 594 for TR, APC for Cy, APC for V6 and V7 fluorescence emission. Flow cytometry data were analyzed with FlowJo software (Tree Star).

■ RESULTS

Fluorescent E4-like neopeptides were created through two different approaches (Figure 2). The initial strategy was based on the direct reaction of E4 with the NHS ester of different fluorophores. Due to multiple reactive amines in the native E4 sequence (N-terminal, K12, K27), amino acid substitutions at the 12 and/or 27 position (K to R) and FMOC protection at the N-terminus were introduced to ensure site specific conjugation. The conjugates were purified by high performance liquid chromatography (HPLC) and the structural identities were confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) with or without trypsin digestion (Figure 3). The second approach utilized azide−alkyne Huisgen cycloaddition using microwave assisted copper catalyzed click-chemistry. An alkyne modified unnatural amino acid, propargylglycine (Pra), was inserted at the 12, 27, or C-terminus of E4 and reacted with an azide modified fluorophore, 11-azido-3,6,9-trioxaundecan-1-amide-Fl. The 11-azido-3,6,9-trioxaundecan-1-amide-Fl was generated by conjugation of 11-azido-3,6,9-trioxaundecan-1-amine to a fluorophore NHS ester in the presence of triethylamine. The azide modified fluorophores were purified by HPLC in good yields (60−80%) and characterized by liquid chromatography−mass spectrometry (LC/MS) analysis. The fluorescent E4-like neopeptides

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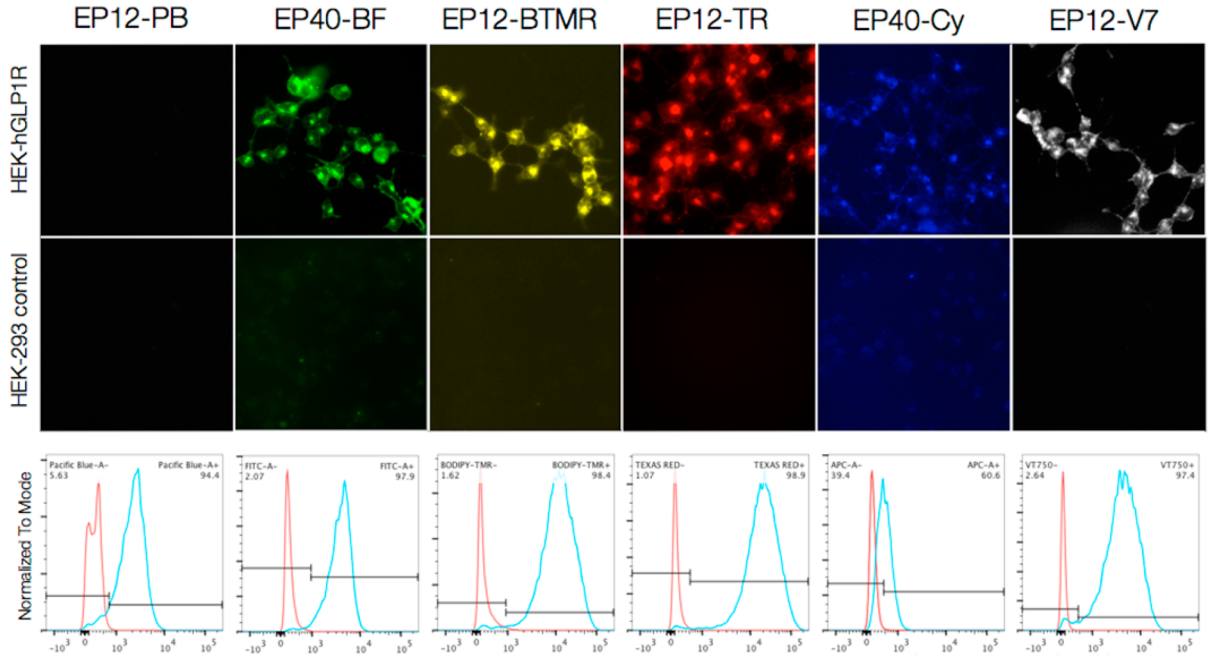

Figure 4. Top: In vitro imaging of the high affinity fluorescent exendin-4 like neopeptides in HEK-hGLP1R cells (top images) or HEK-293 cells (GLP1R negative) (bottom images). Bottom row: The high affinity fluorescent exendin-4 like neopeptides work to different degrees in cell sorting applications. Flow cytometry histogram of single HEK-hGLP1R cells incubated with fluorescent exendin-4 like neopeptide shows a shift in fluorescence (blue line) compared with the HEK-293 (GLP1R negative) incubated cells (red line).

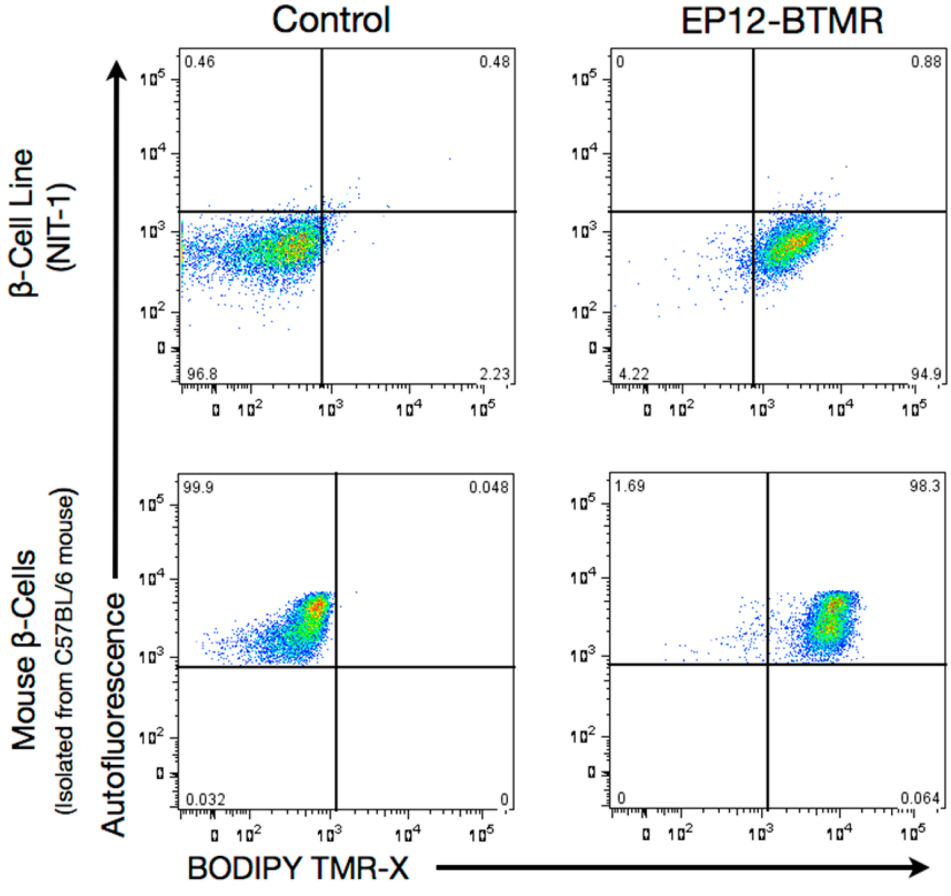

Figure 5. Flow cytometry plots of single channel labeling with fluorescent exendin-4-like neopeptide, EP12-BTMR in a mouse β-cell line (NIT-1), and ex vivo mouse β-cells, demonstrating consistent, specific labeling of β-cells.

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were isolated by HPLC purification and characterized by MALDI-MS (Figure 3).

We first compared the binding affinity of the fluorescent E4-like neopeptides with that of E4 using an I^{125}-exendin-4 (9−39) competitive receptor binding assay in HEK-hGLP1R cells. HEK-hGLP1R cells are an established in vitro human GLP1R model system,[13] are easy to grow, and sufficient numbers of cells can easily be screened in high throughput fashion. Substitution of K12 to R/Pra or addition of an amino acid at position 40 was well tolerated (IC_{50} = 2.2 ± 1.3 nM (**3**), 4.2 ± 1.4 nM (**5**), and 3.3 ± 1.3 nM(7)), while substitution of K27 to R/Pra resulted in 1.5−4-fold lower affinity for GLP1R (IC_{50} = 4.8 ± 1.5 nM (**2**), 6.1 ± 1.4 nM (**4**), and 12.5 ± 1.4 nM (**6**)) than E4 (IC_{50} = 3.2 ± 1.3 nM (**1**)) as shown in Table 1. The intolerance of position 27 for substitution was amplified upon conjugation to BF (IC_{50} = 16.9 ± 1.3 nM (**8**), 35.7 ± 1.3 nM (**9**), and 19.9 ± 1.2 nM (**10**)) and VT750 S (IC_{50} = 61.6 ± 1.2 (**11**) and 96.1 ± 1.3 (**12**)), eliminating the ability to use site specific NHS chemistry for future fluorescent E4-like neopeptides. Amino acid substitution or addition of Pra at the 12 or 40 position was well tolerated and allowed for the investigation of the effect of conjugation at the 12 vs 40 position. There was little to no difference in conjugation at the 12 vs 40 position (Table 1). All fluorescent Pra compounds exhibited dose-dependent receptor binding with the majority maintaining high GLP1R affinity at low nM concentrations (IC_{50} = 3−6 nM (**13**−**22**); see Table 1). However, we noted a 28−35-fold lower affinity with VT680 XL (**23**, **24**).

To determine whether fluorescent E4-like neopeptides could be used for cytometry, we performed additional experiments. Cells (HEK-hGLP1R or HEK-293 as a control) were incubated with fluorescent E4-like neopeptides for 30 min. Confocal imaging of the cells determined that the fluorescent probe could be identified only in cells containing the GLP1R (Figure 4). It was observed that the PB and CyAL 5 conjugates showed low fluorescence, nonspecific binding, and inferior imaging qualities in general. Flow cytometry recorded after incubation of HEK-hGLP1R and HEK-293 cells with fluorescent E4-like neopeptides (**13**−**22**) for 1 h are summarized in Figure 4. The fluorescent E4-like neopeptides labeled GLP1R specific cells (blue line) with no labeling of HEK293 (red line).

To corroborate the above finding using the HEK-hGLP1R cell line, we also performed additional testing in an established mouse β-cell line (NIT-1) as well as in β-cells isolated from mice. Although EP12-BF and EP40-TR both show excellent flow cytometry profiles, EP12-BTMR was chosen for additional experiments based on the availability of the 488 nm laser in most flow cytometry instruments. NIT-1 is a pancreatic β-cell line established from a transgenic NOD/Lt mouse,[14] which is commonly used in diabetes research.[15] Similar staining patterns were observed with NIT-1 cells as for HEK-hGLP1R cells. For example, excellent labeling of NIT-1 cells was observed 30 min after incubation with EP12-BTMR (Figure 5). Similarly, we show that EP12-BTMR was able to stain β-cells from C57BL/6 mice in near quantitative fashion (Figure 5B).

DISCUSSION

In the current study, we synthesized 19 new fluorescent exendin-4 derivatives and tested over 25 exendin-4 neopeptides for their ability to target GLP1R. Using a combination of receptor binding studies, flow analysis, and imaging experiments, we show that there is considerable variability in affinity and, perhaps more importantly, in biological behavior of the different compounds.

Pra substituted E4 (either at the 12 or 40 position) were easy to modify and purify and yielded the most suitable affinity reagents. Modification of the native lysine at the 27 position was not well tolerated, perhaps as expected from theoretical binding studies.[16] It has been observed that the 27 position is involved in a key salt bridge with residue E127(N) of GLP1R.[16] While amino acid substitution with arginine likely maintains the salt bridge, fluorophore conjugates would disrupt this key interaction resulting in a reduced binding affinity. We also observed considerable differences between the different fluorochromes, particularly in binding affinity, in vitro imaging, and cell labeling studies. Overall, EP40-BF, EP12-BTMR, and EP12-TR have good affinity, ideal imaging properties, and specific GLP1R labeling, making them ideal probes for multicolor β-cell flow cytometry analysis.

The current study shed some light on the design of a future generation of E4 peptides for flow cytometry and microscopy. We identified some highly promising compounds, but invariably new fluorochromes may lead to even better profiles. Using the current affinity reagents, we predict that, collectively, these compounds will be particularly useful for β-cell research, rapid β-cell analysis, and in vitro imaging. These studies are all the more important since it was recently discovered that the commercially available GLP1R antisera commonly used are not as reliable for the detection of the GLP1R protein as previously believed.[2] We thus believe that the fluorescent E4s developed will be useful for basic and translational β-cell research.

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Notes

The authors declare no competing financial interest.

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